Journal of Medical Virology 55:75–81 (1998)

Glycoprotein B Genotype Correlates With Cell Tropism In Vivo of Human Cytomegalovirus Infection

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Human cytomegalovirus (HCMV) strains can be classified into four genotypes of the glycoprotein B (gB). In a previous study, the gB genotype 1 was found more frequently in bone marrow transplant recipients with nonfatal HCMV infection than in patients who died from HCMV disease [Fries et al. (1994): Journal of Infectious Diseases 169:769–774]. The distribution and cell tropism of different gB types in vivo were investigated. The gB type of HCMV was determined in blood or urine specimen from 76 organ and 47 bone marrow transplant recipients using PCR and restriction fragment length polymorphism (RFLP). The leukocyte populations (polymorphonuclear leukocytes, monocytes, T lymphocytes, non-T lymphocytes) of 20 viremic patients were purified by a fluorescence-activated cell sorter (FACS) and examined for HCMV infection by PCR. Sequence analysis of four randomly selected strains showed that gB types were similar to published sequences and no atypical gB types were found. Within the compartments blood and urine, the gB types were almost equally distributed, whereas the gB type 1, in contrast to gB types 2 and 3, did not infect T lymphocytes in vivo. These data show that the gB type correlates with viral tropism in vivo and thus provides further evidence that the gB variation may indeed influence the virulence of HCMV. ***J. Med. Virol. 54:75–81, 1998.***

KEY WORDS: HCMV; gB genotype; tropism

INTRODUCTION

In immunocompetent individuals, infection with the human cytomegalovirus (HCMV) is generally asymptomatic. In contrast, HCMV causes significant morbidity and mortality in immunocompromised patients. However, not all immunocompromised patients with HCMV infection develop HCMV-associated disease. Bone marrow transplant recipients, who develop a cytotoxic T-cell response to HCMV, are known to be less susceptible to HCMV disease [Reusser et al., 1991]. Thus, obviously, the immunological status of the patient influences the clinical outcome of HCMV infection. It has also been proposed that different virus strains may vary in virulence [Fries et al., 1994; Brown et al., 1995].

HCMV strains are known to differ in their ability to infect lymphocytes and hematopoietic progenitor cells [Einhorn and Öst, 1984; Rakusan et al., 1989; Rice et al., 1984; Simmons et al., 1990]. Some isolates inhibit hematopoiesis in vitro more efficiently than others [Simmons et al., 1990]. Differences in the ability to replicate in SCID-hu mice have also been observed [Brown et al., 1995]. On the nucleotide level, the homology between different isolates varies between 50% and 100%, depending on the region analyzed [Bale et al., 1993; Lehner et al., 1991].

Highly variable regions are found in the glycoprotein B (gB) gene of HCMV. This protein not only is the major target for neutralizing antibodies [Cranage et al., 1986], but is also important for the entry of virions into cells, transmission of infection from cell to cell, and the fusion of infected cells [Navarro et al., 1993; Tugizov et al., 1994; Tugizov et al., 1995]. The highly variable region around the protease cleavage site (between amino acid 460 and 461) is involved in these processes [Navarro et al., 1993]. Based on the nucleotide sequence coding for this variable region, a genotyping scheme was proposed and HCMV strains were classified into

Contract grant sponsor: Bundesministerium für Forschung und Technologie; Contract grant number: 01 KI 9303.

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Accepted 28 October 1997

four gB types [Chou, 1992]. In bone marrow transplant recipients, infection with gB type 1 virus was found to correlate with a nonfatal clinical outcome, while gB types 2, 3, and 4 were found more frequently in patients who died of HCMV-associated disease [Fries et al., 1994]. In patients with acquired immunodeficiency syndrome (AIDS), the gB type 1 was found less frequently in patients with retinitis than in asymptomatic AIDS patients [Bongarts et al., 1996; Rasmussen et al., 1995]. Furthermore, symptomatic HCMV infection is closely linked with viremia [Meyer-König et al., 1995b]. We therefore investigated whether the gB genotypes differ in their tropism for peripheral blood leukocytes (PBL) in vivo. The distribution of gB genotypes in PBL and urine as well as in different leukocyte populations was determined in bone marrow and organ transplant recipients.

MATERIALS AND METHODS

Patients

A total of 184 blood and 38 urine samples from immunosuppressed patients were examined. Forty-seven bone marrow, 60 kidney, eight liver, and eight heart transplant recipients were included in this study. The patients were monitored for the presence of HCMV DNA by polymerase chain reaction (PCR) and for the pp65 antigen by indirect immunofluorescence using peripheral blood leukocytes (PBL). Specimens were obtained weekly from patients hospitalized after transplantation (Tx) and at more irregular intervals from outpatients during followup. Patients were followed for at least six months after transplantation.

Processing of Specimens

For PCR analysis and pp65 antigen detection, the PBL were separated using dextran and hypotonic lysis of erythrocytes as described [van der Bij et al., 1988]. A total of 2×10^5 PBL were used for the pp65 antigen test by indirect immunofluorescence as described previously [Meyer-König et al., 1995a].

For FACS-sorting, PBL (from 20-ml blood) were separated using Ficoll-Paque gradient centrifugation (Pharmacia density medium; Uppsala, Sweden). Blood mononuclear cells (PBMC) were stained with monoclonal antibodies anti-CD3 (UCHT-1FITC, Immunotech, Marseilles, France) and anti-CD14 (IOM2PE, Immunotech). In some patients, PBMC were stained additionally with anti-CD4 and anti-CD8 (Leu3a and Leu2a, Becton Dickinson, San Jose, CA). The cells in the pellet of the Ficoll gradient (polymorphonuclear leukocytes, PMNL, and erythrocytes) were resuspended in erythrocyte lysing buffer [van der Bij et al., 1988] and then washed three times with phosphate-buffered saline (PBS). The remaining enriched polymorphonuclear leukocytes (PMNL) were stained with anti-CD16 (ION16, Immunotech).

Cells were sorted on a FACStar plus cell sorter (Becton Dickinson). $CD3^-$ (CD3-negative) non-T lymphocytes (mostly B lymphocytes and NK cells) were sorted from Ficoll-purified mononuclear cells according to their low forward and side light scatter and negative staining for CD3. The $CD14^+$ monocytes and the $CD3^+$ and $CD3^-$ lymphocyte fractions had a purity of >98%. The sorted $CD16^+$ PMNL had a purity of >99.5%.

DNA Preparation

Total cells (2×10^6 cells) and purified cell fractions (10^3 to 10^5 cells, each) were resuspended in a PCR-compatible lysis buffer (10-mM Tris at pH 8.3, 2.5-mM $MgCl_2$, 50-mM KCl, 0.5% Tween 20, 0.5% NP-40; 250 μl for total cells and 75 μl for purified cell fractions) with 0.2-mg/ml proteinase K. After incubation (60°C, 120 min), the proteinase K was inactivated (95°C, 10 min). The crude DNA extract was used for PCR amplification. The DNA of 200-μl urine was extracted using the Qiamp Blood Kit (Quiagen, Hildeu, Germany) according to the manufacturer's recommendations.

PCR Amplification of Sequences of pp150 and Immediate Early (IE) Gene

Two different nested PCR reactions that amplified HCMV DNA from the pp150 gene and the IE1 gene were carried out as described previously [Meyer-König et al., 1995b; von Laer et al., 1995]. PCR detected less than five copies of plasmid DNA.

PCR Amplification of gB Gene and Restriction Analysis

A region of high peptide variability in the gB gene was amplified as described previously [Fries et al., 1994]. A first round of amplification was added to improve sensitivity. To overcome sequence variation between strains, a mixture of three 5′ primers was used (5′ gBout1: 5′-GTTCCGAAGCCGAAGACTCG; 5′ gBout2: 5′-GCAGCACCTGGCTCTATCG; 5′ gBout3: 5′-GCCAGCTCACCTTCTGG; 3′ gBout: 5′-GCACCTTGACGCTGGTTTGG). The first round of amplification was carried out in a total volume of 50 μl using crude DNA extract and PCR mix A (10-mM Tris-Cl at pH 8.3, 2.5-mM $MgCl_2$, 50-mM KCl, 0.01% gelatin, 400 μM each of the four dNTPs, 1.25 units of Taq polymerase (Promega, Madison, WI) with 5 pmol of each 5′ primer and 15 pmol of the 3′ primer. Second-round amplification was carried out in a total volume of 50 μl, adding 1 μl of first-round amplification product to 49 μl of PCR mix B (10-mM Tris at pH 8.3, 2.5-mM $MgCl_2$, 50-mM KCl, 0.01% gelatin, 200 μM each of the four dNTPs, 1.25 units of Taq polymerase) supplemented with 5 pmol of each primer. The samples were covered with mineral oil (Sigma, Deisenhofen, Germany), denatured (95°C, 360 sec), and amplified within 35 cycles (95°C, 90 sec; 55°C, 120 sec; 72°C, 60 sec). Amplification products (293 to 296 bp, size varies by strain) were analyzed by electrophoresis in a 2% agarose gel stained with 0.5-μg/ml ethidium bromide and subjected to restriction analysis using *Hinf* I and *Rsa* I (New England Biolabs, Beverly, MA) as described [Fries et al., 1994]. Digested DNA was analyzed on 10% polyacrylamide gels. Four distinct gB genotypes were

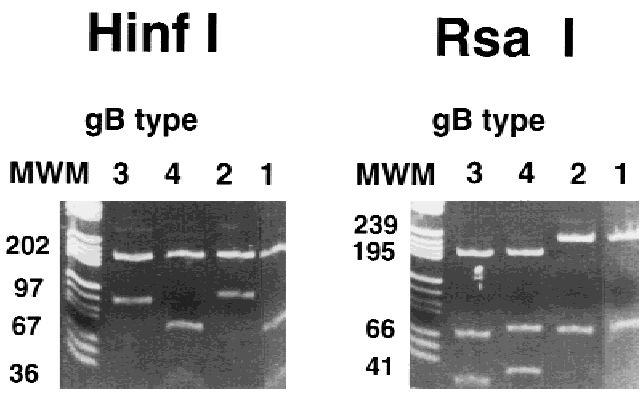

Fig. 1. Restriction fragment analysis for gB typing. Four distinct gB genotypes were identified by the different length of restriction fragments (36 to 239 bp) using *Hinf* I and *Rsa* I. MWM denotes molecular weight marker.

identified by the different lengths of restriction fragments (36 to 239 bp) (Fig. 1).

Sequencing Strategy

The second-round amplification products of the gB PCR were purified (Qiaquick spin PCR purification kit, Quiagen) and 100 ng of DNA were used for sequencing reactions according to the dideoxy chain termination technique (Prism ready reaction dyedeoxy terminator cycle sequencing kit, Perkin Elmer, Norwalk, CT). Completed sequencing reactions were loaded on a 6% polyacrylamid gel and analyzed by an automated DNA sequencer (ABI Model 373, Applied Biosystems, Weiterstadt, Germany). Sequence similarity was analyzed by the Jotun Hein method [Brew et al., 1996].

Statistics

Significance was determined using the χ^2-test.

RESULTS

Sequence Similarity of HCMV gB Strains

To rule out geographical differences, four gB strains obtained in Freiburg were sequenced and compared with data published originally in the Portland study [Chou and Dennison, 1991]. Direct sequencing between codon 449–532 was performed. This corresponded to the region amplified by gB PCR and used for restriction enzyme digestion (bases 1347–1597). Table I shows that sequence similarity within one gB group was >99% as published.

Distribution of gB Types in Bone Marrow Transplant and Organ Transplant Recipients

The gB genotype of HCMV was determined in blood and/or urine specimens of immunosuppressed patients. In the majority of patients only one gB type was found. Figure 2 shows the frequency distribution of gB types and mixed types in organ and bone marrow transplant recipients. The gB type 2 was almost equally distributed in these populations ($P = 0.55$), whereas the gB type 1 was found less frequently in organ transplant recipients ($P = 0.027$) and the gB type 3 was found less frequently in bone marrow transplant recipients ($P = 0.026$). Only a few patients were infected with the gB type 4.

A mixture of gB types was found in 14 of 123 patients and was classified as follows. Different gB types were found, (one), in sequential samples of one patient (5 of 14 patients); (two), in different compartments (blood/urine) of one patient (4 of 14 patients); and (three), in the same sample of one patient (5 of 14 patients). In the last case, restriction analysis produced fragments, which corresponded to two gB types. These double infections could be resolved into the known gB types analyzing tenfold dilutions of DNA extracts by PCR. Each dilution was tested fourfold. The dilution step positive in two of four samples was then used for gB typing (20 reactions were typed). A typical example is given in Figure 3.

Blood and urine samples were obtained from 30 of the 123 patients. The gB types 1 to 4 were almost equally distributed in these compartments (blood and urine) (data not shown).

Distribution of gB Types in Primary and Reactivated HCMV Infection

Clinical information and pretransplant HCMV serology were available from 113 of the 123 patients. Primary HCMV infection and reactivation of infection were found in 26 and 43 of 69 organ transplant recipients, respectively. In bone marrow transplant recipients, primary HCMV infection and reactivation of infection were found in 6 and 38 of 43 patients, respectively. Table II shows that HCMV gB types were distributed equally in primary infection and reactivation.

Distribution of gB Types in Leukocyte Populations

We then investigated whether the gB types are distributed equally in all leukocyte populations. Blood samples from 14 organ transplant and six bone marrow transplant recipients were examined. The PBL of these patients were found positive either in pp65 antigen test or HCMV PCR (amplification of the IE and the pp150 gene). Seven patients had a primary HCMV infection, whereas 13 patients had a reactivation of infection. The blood samples were used for gB typing and the following leukocyte populations were purified by FACS: $CD16^+$ polymorphonuclear leukocytes (PMNL), $CD14^+$ monocytes, $CD3^+$ T lymphocytes, and $CD3^-$ non-T lymphocytes (mostly B lymphocytes and NK cells). The purity was >98% for monocytes and lymphocytes and >99.5% for granulocytes. To estimate the viral load, tenfold dilutions (10^5, 10^4, and 10^3 cells) of the purified cell fractions were then analyzed for HCMV DNA by PCR. The minimal number of PCR-positive cell are given in Table III. If a positive signal only could be obtained using 10^5 cells and PCR failed to detect HCMV DNA in 10^4 cells, this corresponded to an infection rate of approximately 0.001% (detection of DNA in

TABLE I. Sequence Homology Among HCMV Strains of the Present Study as Compared to Published Data

	US-gB1[a]	US-gB2[a]	US-gB3[a]	US-gB4[a]	Fr-gB1[b]	Fr-gB2[b]	Fr-gB3[b]	Fr-gB4[b]
US-gB1	100%	95%	82%	93%	100%	95%	81%	93%
US-gB2		100%	81%	89%	95%	100%	81%	89%
US-gB3			100%	81%	82%	82%	99%	81%
US-gB4				100%	93%	89%	80%	100%

[a]Sequence homology (bases 1347–1597) of published gB strains 1 to 4 [Chou and Dennison, 1991]: US-gB1 (C327), US-gB2 (AD 169), US-gB3 (C359), and US-gB4 (C194).
[b]Sequence homology (bases 1347–1597) of gB strains 1 to 4 obtained in Freiburg (Fr-gB1 to 4).

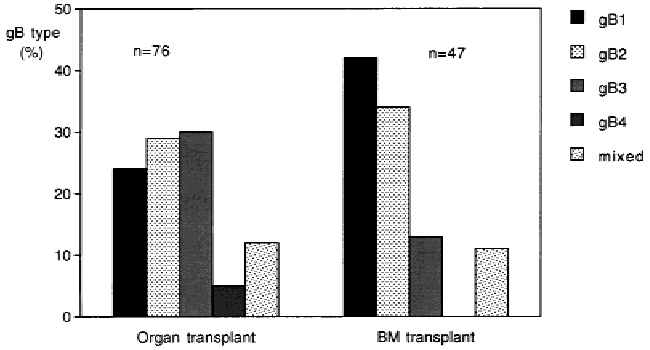

Fig. 2. Distribution of gB types in bone marrow and organ transplant recipients. The gB genotype of human cytomegalovirus (HCMV) was determined in blood and urine specimen of 76 organ transplant recipients and 47 bone marrow (BM) transplant recipients using nested polymerase chain reaction (PCR) and restriction analysis as described in Materials and Methods.

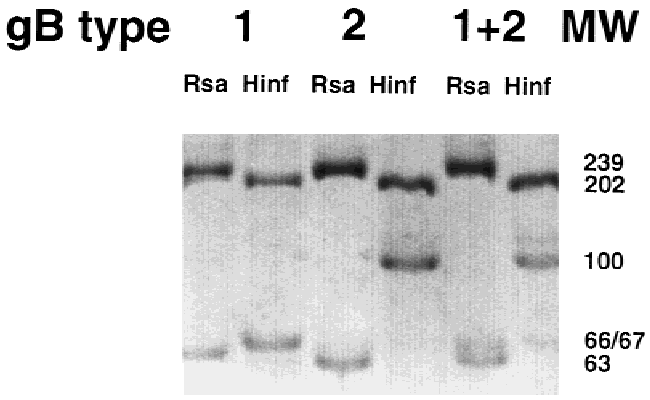

Fig. 3. Analysis of gB types in mixed infections. The restriction analysis of the blood sample shown did not allow a clear diagnosis of the gB type (shown under "1 + 2"). The DNA was diluted and eight out of 20 of these diluted samples were gB PCR-positive. The amplification products were then subjected to restriction analysis. The pattern was now typical for gB type 1 in five of the diluted samples (one example shown under "1") and for gB type 2 in three of the diluted samples (one example shown under "2"). MW denotes molecular weight in kDa.

10^4 cells corresponding to 0.01%, detection of DNA in 10^3 cells to 0.1%).

HCMV DNA was not detected in the T lymphocytes of 10 patients with gB type 1 viremia. In contrast, nine out of 10 patients infected with gB types 2 or 3 harbored HCMV in their T lymphocytes. Thus, the gB types 2 and 3 were T lymphotropic in vivo and the gB type 1 virus was not ($P < 0.0001$).

TABLE II. Distribution of gB Types in Primary Infection and Reactivation

Type	Primary HCMV infection	Reactivation of HCMV infection
gB type 1	9 (28%)	27 (33%)
gB type 2	9 (28%)	28 (35%)
gB type 3	10 (31%)	17 (21%)
gB type 4	1 (3%)	4 (5%)
Mixed types	3 (9%)	5 (6%)
Totals	32	81

In five patients infected with gB types 2 or 3 $CD4^+$ and $CD8^+$, T lymphocytes were examined separately. Two samples were HCMV DNA-positive in the $CD8^+$ cells only, one in the $CD4^+$ cells only, and two samples were HCMV-positive in both T lymphocyte subpopulations (data not shown). The gB types 2 and 3 were also found more frequently in non-T lymphocytes than the gB type 1 ($P = 0.04$). In most patients, the $CD14^+$ monocytes were HCMV DNA-positive; and in all patients, viral DNA was detected in the PMNL. The patients studied were rarely infected with gB type 4 virus, and the tropism of this genotype for leukocyte populations in vivo could not be examined.

The gB type was then determined in the purified leukocyte populations from a patient infected with both gB type 1 and gB type 2 virus. In the PMNL, monocytes, and non-T lymphocytes, a mixture of gB type 1 and type 2 sequences were amplified. In contrast, only gB type 2 virus was detected in the T lymphocytes. This result again shows that gB type 2 virus is T lymphotropic in vivo, whereas gB type 1 virus is not.

DISCUSSION

The distribution and cell tropism of HCMV gB genotypes were examined in samples from bone marrow and organ transplant recipients. A highly variable region around the cleavage site of the gB gene of four randomly selected HCMV strains was sequenced and it was shown that gB types were similar to published sequences [Chou and Dennison, 1991]. No atypical gB types were found.

The analysis of blood and urine samples showed that the gB genotype was almost equally distributed in different compartments. The gB type, however, was found to correlate with T-cell tropism of HCMV in vivo. The gB type 1 virus did not infect T lymphocytes. In con-

TABLE III. Detection of HCMV DNA in FACS-Purified Leukocyte Populations

Patient number	Tx[a]	P/R[b]	pp65[c]	gB type	Cell fraction[d]			
					$CD3^+$	$CD3^-$	CD14	CD16
1	ORtx	P	28	1		10^4	10^4	10^3
2	ORtx	R	2	1			10^5	10^5
3	ORtx	R	0	1			10^5	10^5
4	ORtx	R	23	1		10^5		10^5
5	ORtx	R	21	1				10^5
6	ORtx	R	4	1				10^4
7	ORtx	R	8	1			10^3	10^3
8	BMtx	R	3	1				10^4
9	BMtx	R	27	1			10^3	10^4
10	BMtx	R	4	1				10^4
11	ORtx	P	1	2	10^5	10^5		10^5
12	ORtx	P	182	2	10^4	10^5	10^4	10^3
13	ORtx	P	130	3	10^5		10^5	10^3
14	ORtx	P	10	3	10^5	10^3	10^4	10^3
15	ORtx	P	7	3			10^5	10^4
16	ORtx	P	7	3	10^5	10^3		10^5
17	ORtx	R	20	3	10^5		10^3	10^4
18	BMtx	R	34	3	10^5	10^5	10^5	10^5
19	BMtx	R	10	3	10^5	10^5	10^5	10^3
20	BMtx	R	1152	3	10^4	10^4	10^5	10^3

[a]Patients after organ transplantation (ORtx) or bone marrow transplantation (BMtx).
[b]Patients with primary (P) or reactivated (R) HCMV infection.
[c]Number of pp65 antigen-positive cells/2 × 10^5 leukocytes.
[d]Tenfold dilutions of 10^5 FACS-purified cells (10^5, 10^4, and 10^3 cells) were tested for HCMV DNA by PCR and the minimal number of cells positive in the PCR is given. Negative PCR (–).

trast, the gB type 2 and 3 viruses infected lymphocytes as well as granulocytes and monocytes. The more expanded tropism of gB type 2 and 3 viruses was not due to a higher viral load as compared to gB type 1 infections. This difference was also found in patients with low viral load (Table III, patients number 11, 14, 16, and 19 with $\leq$10 pp65 antigen-positive cells/2 × 10^5 leukocytes).

Furthermore, it could be argued that the infection of T lymphocytes was due to contaminating granulocytes or monocytes. It is unlikely, however, that DNA-positive granulocytes or monocytes contaminated T lymphocyte fractions only in patients with gB type 2 or 3 viremia and not in patients with gB type 1 viremia, since purity of cell fractions was equal in all samples.

No correlation of the HCMV gB genotype with the frequency of primary or reactivated infection, nor with the clinical outcome of infection, was found in this study. This may be due to the fact that all patients received antiviral therapy as soon as HCMV PCR and/or the pp65 antigen test became positive. The apparent prevalence of reactivation in gB type 1 infections of patients analyzed by FACS (Table III) is a distorted picture, since no such prevalence was found within the whole population of patients studied (Table II).

Previous studies have shown that the gB genotype is correlated with the course of HCMV disease in bone marrow transplant recipients and AIDS patients [Bongarts et al., 1996; Fries et al., 1994; Rasmussen et al., 1995; Shepp et al., 1996]. Obviously, the type of cells and tissues that are infected can determine the clinical outcome of virus infections. In the case of HSV-1, gB variation has been shown to influence pathogenicity for mice. One single amino acid substitution lead to enhanced neuroinvasiveness [Goodman and Engel, 1991; Weise et al., 1987]. Furthermore, in HIV infection, T-cell and macrophage tropic strains can be distinguished [Shioda et al., 1991]. This contributes to a different clinical outcome as infection with macrophage tropic strains frequently leads to brain damage [Hein and Stovlbaek, 1996]. Therefore, the correlation of HCMV gB type and cell tropism in vivo supports the hypothesis that the gB variation may influence the pathogenicity of HCMV.

However, for several reasons we believe that the preferential tropism of the gB types 2 and 3 for T lymphocytes cannot be the only mechanism by which the gB type influences clinical outcome. In previous studies, the viral load in T lymphocytes was generally found to be low [Einhorn and Öst, 1984; Gerna et al., 1992; Nelson et al., 1990; Numazaki et al., 1994; Rice et al., 1984; Schrier et al., 1985; von Laer et al., 1995]. Also in this study, less than 0.1% of the T lymphocytes were infected. Thus, the disturbed function of T lymphocytes during HCMV infection is most likely not simply due to a direct cytopathic effect of HCMV [Müller and Einsele, 1992]. Furthermore, infection of T lymphocytes is not expected to contribute substantially to the viral load in vivo, since monocyte and polymorphonuclear leukocytes are the major target for HCMV during viremia [Dankner et al., 1990; von Laer et al., 1995]. Therefore, our data provide additional evidence that gB types behave differently in vivo, but do not yet fully explain why the gB type correlates with viral pathogenicity.

The question arises, of whether the gB protein itself influences the tropism and virulence of HCMV, or whether the gB gene is genetically linked to some other trait, which is the actual virulence marker. The gB

genotype is known to be linked to the genotype of another membrane protein of HCMV, the gH protein. However, the gH genotype was not found to correlate with clinical outcome [Fries et al., 1994]. Therefore, the differences observed in tropism and virulence of different gB types in vivo may well be determined by the gB protein itself and due to one of the known properties of the gB protein: promotion of viral entry into cells and spread of virus from cell to cell [Navarro et al., 1993; Tugizov et al., 1994; Tugizov et al., 1995].

Considering these functions of gB, the most obvious explanation for the preferential tropism of the gB types 2 and 3 for T lymphocytes would be that the gB proteins differ in their ability to support penetration of virions into T lymphocytes. On the other hand, it was shown that HCMV-infected fibroblasts upregulate lymphocyte adhesion molecules [Grundy et al., 1993; Grundy and Downes, 1993; Sedmak et al., 1994]. Therefore, T lymphocytes could well be infected by direct cell-to-cell transmission of HCMV and gB strains might differ in their ability to regulate and interact with adhesion molecules. A more efficient transmission of gB types 2 and 3 viruses could also explain why T lymphocytes are preferentially infected by these virus types in vivo. A more efficient cell-to-cell spread of the gB types 2 and 3 would also explain their higher virulence. Further in vitro studies will be necessary to clarify why T lymphocytes are preferentially infected by gB 2 and 3 genotypes in vivo.

In conclusion, it was demonstrated that the gB types of HCMV differ in their tropism in vivo. Because viral tropism and pathogenicity are frequently linked, the results provide further evidence for the hypothesis that the gB variation influences the virulence of HCMV.

ACKNOWLEDGMENTS

We thank O. Haller and D. Neumann-Haefelin for helpful discussions and critical reading of the manuscript. We also thank C. Binder and K. Musiol for excellent technical assistance.

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